The Aryl Hydrocarbon Receptor Is Involved in UVR-Induced Immunosuppression

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UVR suppresses the immune system through the induction of regulatory T cells (Tregs). UVR-induced DNA damage has been recognized as the major molecular trigger involved, as reduction of DNA damage by enhanced repair prevents the compromise to the immune system by UVR. Nevertheless, other signaling events may also be involved. The aryl hydrocarbon receptor (AhR) was identified as another target for UVR, as UVR activates the AhR and certain UVR effects were not detected in AhR-deficient cells. We studied whether the AhR is involved in UVR-induced local immunosuppression and whether similar effects can be induced by AhR agonists. The AhR antagonist 3-methoxy-4-nitroflavone reduced UVR-mediated immunosuppression and the induction of Tregs in murine contact hypersensitivity (CHS). Conversely, activation of the AhR by the agonist 4-n-nonylphenol (NP) suppressed the induction of CHS and induced antigen-specific Tregs similar to UVR. This was further confirmed in AhR knockout mice in which UVR- and NP-induced immunosuppression were significantly reduced. Together, this indicates that the AhR is involved in mediating UVR-induced immunosuppression. Activation of the AhR might represent an alternative to modulate the immune system in a similar manner like UVR but without causing the adverse effects of UVR, including DNA damage.

Journal of Investigative Dermatology (2013) **133,** 2763–2770; doi:10.1038/jid.2013.221; published online 11 July 2013

INTRODUCTION

UVR, in particular the mid-wave range (UVB, 290–320 nm), suppresses the immune system. UVR-induced immunosuppression exerts unique features: (1) it is caused by low suberythemogenic doses; (2) it is antigen specific and thus differs from immunosuppression caused by drugs; and (3) primarily, T cell–driven reactions are affected (Schwarz, 2010). The antigen specificity is due to the generation of regulatory T cells (Tregs). Because of their specificity, UVR-Tregs harbor therapeutic potential. Hence, we are interested to characterize these cells and try to elucidate the mechanisms by which they are induced.

UVR-induced DNA damage appears to be an essential molecular trigger for the induction of Tregs, as upon reduction of DNA damage, Tregs are not generated (Schwarz *et al.*, 2005). Unless completely removed by the nucleotide excision repair, UVR-induced DNA damage bears the risk for malignant transformation. This is the major drawback of utilizing UVR as a therapeutic tool. To overcome this risk, we are searching for alternative strategies to induce Tregs, which act in the same manner like UVR-Tregs. Recently, we showed that injection of antimicrobial peptides may be such a route (Navid *et al.*, 2012).

The aryl hydrocarbon receptor (AhR) is a ubiquitously expressed ligand-activated transcription factor involved in the detoxification of aromatic hydrocarbons. The AhR, however, appears to also mediate other cellular events such as proliferation, cell growth, and differentiation (Abel and Haarmann-Stemmann, 2010). Different low-molecular-weight ligands bind to the AhR and act in an agonistic or antagonistic manner, thereby modulating AhR activation. Classical ligands of the AhR are exogenic xenobiotics such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), benzo(a)pyrene, and 3-methylcholanthrene (Denison and Nagy, 2003). After activation, the AhR translocates into the nucleus where it associates with the AhR nuclear translocator. This heterodimer then binds to xenobiotic response elements of a variety of genes involved in the cellular detoxification pathway of xenobiotics (Bock and Köhle, 2006). One important target gene is *CYP1A1*, which is widely used as a marker for AhR activation (Fritsche *et al.*, 2007; Hu *et al.*, 2007; Abel and Haarmann-Stemmann, 2010). However, the biological consequences of AhR activation exceed detoxification processes.

The AhR was recently recognized to serve as an intracellular molecular target for UVR (Fritsche *et al.*, 2007).

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Abbreviations: AhR, aryl hydrocarbon receptor; BMDC, bone marrow–derived dendritic cell; CHS, contact hypersensitivity; DEREG, DEpletion of REGulatory T cells; DT, diphtheria toxin; Foxp3, forkhead box P3; i.p., intraperitoneal; KO, knockout; LC, Langerhans cell; MNF, 3-methoxy-4-nitroflavone; NP, 4-n-nonylphenol; Oxa, oxazolone; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; Treg, regulatory T cell; UVR-Treg, UVR-induced Treg

Received 31 January 2013; revised 5 April 2013; accepted 17 April 2013; accepted article preview online 7 May 2013; published online 11 July 2013

UVR exposure of HaCaT cells induced translocation of the AhR into the nucleus and, subsequently, transcription of *CYP1A1*. Induction of *CYP1A1* by UVR was not observed in AhR knockout (KO) cells, indicating the crucial role of AhR in this signaling event.

In addition, it was observed that the AhR appears to be involved in the induction of Tregs (Quintana *et al.*, 2008). Owing to this fact and the observation that the AhR can be activated by UVR, we asked whether the AhR signaling pathway is involved in UVR-induced immunosuppression and, if this is the case, whether similar immunosuppressive effects can be induced by AhR ligands.

RESULTS

The AhR antagonist MNF reduces UVR-induced immunosuppression

To clarify whether the AhR is involved in UVR-induced immunosuppression, the murine model of contact hypersensitivity (CHS) was used, in which UVR inhibits sensitization and induces antigen-specific Tregs. To block the AhR, the specific AhR antagonist 3-methoxy-4-nitroflavone (MNF; Lu *et al.*, 1995; Davis *et al.*, 2003) was selected. Mice were exposed to UVR on their backs for 4 consecutive days. 24 Hours after the last exposure mice were sensitized against DNFB. One group received MNF intraperitoneally (i.p.) 1 hour before sensitization. After 5 days, mice were challenged, and after further 24 hours ear swelling was measured. Sensitization and challenge with DNFB induced pronounced ear swelling in positive control mice (Figure 1a; #1). The CHS reaction was significantly reduced on UVR exposure (Figure 1a; #3). In contrast, injection of MNF significantly reduced UVR-induced immunosuppression (Figure 1a; #4). MNF alone had no effect on CHS (Figure 1a; #5).

To study whether MNF prevents the induction of Tregs by UVR, adoptive transfer experiments were conducted. Lymph node cells and splenocytes were isolated from mice that were UVR exposed (Figure 1a; #3), or in addition treated with MNF (Figure 1a; #4) and injected into naive mice. After 24 hours, recipients were sensitized and 5 days later challenged with DNFB. The ear swelling response in recipients injected with cells isolated from only UV-irradiated donors was significantly reduced, suggesting that cells with regulatory properties had developed in the donors (Figure 1b; #3). In contrast, CHS was not suppressed in animals injected with cells obtained from UVR-exposed and MNF-treated mice, indicating that antagonizing the AhR prevents UVR-mediated induction of Tregs (Figure 1b; #4).

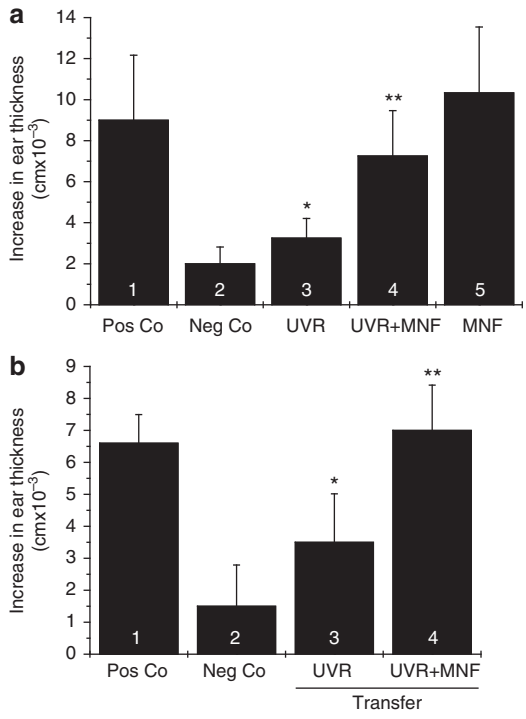

Figure 1. The 3-methoxy-4-nitroflavone (MNF) reduces UVR-induced immunosuppression. (**a**) Mice were irradiated with UVR on 4 consecutive days (#3). At 24 hours after the last irradiation, mice were sensitized with DNFB through UVR-exposed skin. After 5 days, ears were challenged with DNFB, and ear swelling was measured 24 hours later. One group of UVR-exposed mice received MNF (20 μM, 200 μl) intraperitoneally (i.p.; #4) 1 hour before every UVR exposure. As a control, unirradiated mice were injected with MNF alone (#5). Positive control (Pos Co) mice were sensitized and challenged, and negative control (Neg Co) mice were only challenged. Bars show mean ± SD of increase in ear thickness expressed as the difference between the thicknesses of the challenged ear compared with the vehicle-treated ear. *$P<0.05$ versus Pos Co; **$P<0.05$ versus UVR. (**b**) Lymph node cells and splenocytes were obtained from the UVR and UVR + MNF groups (**a**, #3 and #4), respectively, and injected intravenously (i.v.) into naive mice (Transfer) 24 hours before sensitization. After 5 days, ears were challenged and ear swelling measured after 24 hours. *$P<0.005$ versus Pos Co; **$P<0.005$ versus UVR.

Inhibition of the AhR downregulates the suppressive activity of UVR-Tregs

The adoptive transfer experiments implied that blocking of the AhR inhibits the development of UVR-Tregs. However, one cannot exclude that MNF blocks their suppressive activity. To clarify the latter issue, splenocytes and lymph node cells were obtained from mice that were sensitized through UVR-exposed skin. Cells were separated into $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells through magnetobead separation. The latter fraction that contains UVR-Tregs was incubated *in vitro* with MNF. After 24 hours, cells were harvested, washed, and injected intravenously into naive mice that were DNFB sensitized 24 hours later. The ear swelling response revealed that, in contrast to untreated UVR-Tregs (Figure 2a; #3), UVR-Tregs incubated with MNF were less effective in inhibiting CHS in the recipients (Figure 2a; #4). In addition, the incubation of $CD4^+CD25^+$ cells with MNF caused a reduction in the expression of Foxp3 (forkhead box P3; Figure 2b). These data indicate that antagonizing the AhR reduces the expression of Foxp3, which appears to be associated with a mitigation of the suppressive activity of UVR-Tregs.

The AhR agonist NP induces immunosuppression

The data obtained so far suggest that the AhR is involved in mediating UVR-induced immunosuppression. We next checked whether mere activation of the AhR can exert similar immunosuppressive features as UVR. To activate the

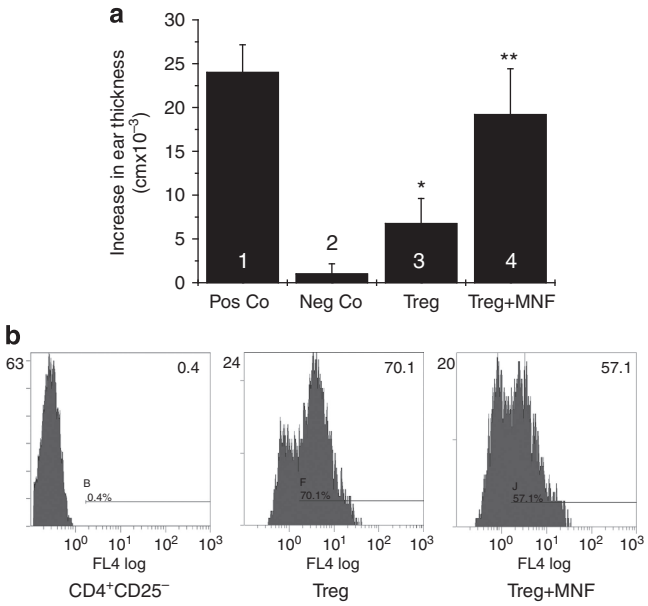

Figure 2. The 3-methoxy-4-nitroflavone (MNF) downregulates the suppressive activity and the expression of forkhead box P3 (Foxp3) by UVR-regulatory T cells (Tregs). (**a**) Mice were sensitized with DNFB through UVR-exposed skin. After 5 days, lymph node cells and splenocytes were obtained and separated into CD4$^+$CD25$^+$ Tregs and CD4$^+$CD25$^-$ T cells. Tregs were incubated with 20 μM MNF (#4) or left unstimulated (#3). After 24 hours, cells were washed and injected intravenously (i.v.; 5×10^5) into naive mice that were sensitized and challenged with DNFB. *P<0.00005 versus Pos Co; **P<0.005 versus Tregs. Neg Co, negative control; Pos Co, positive control. (**b**) Lymph node cells and splenocytes were obtained from UVR and DNFB-treated mice and separated into CD4$^+$CD25$^-$ T cells, CD4$^+$CD25$^+$ (Tregs), and Tregs incubated with MNF. Cells were stained with an antibody directed against Foxp3 and subjected to FACS analysis. Histograms show cell number (y axis) versus fluorescence intensity (x axis).

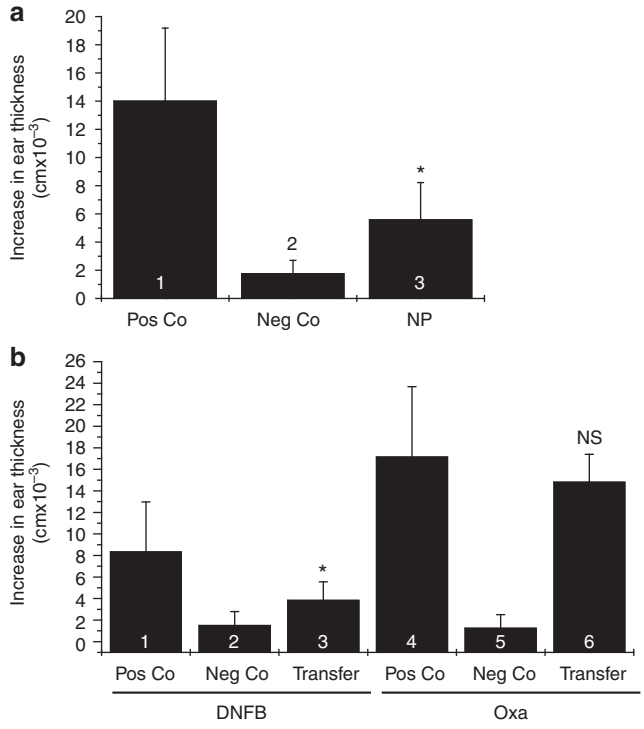

Figure 3. The aryl hydrocarbon receptor (AhR) agonist 4-n-nonylphenol (NP) induces immunosuppression. (**a**) Mice were injected intraperitoneally (i.p.) with 600 nM NP (200 μl) for 4 consecutive days (#3). After 24 hours, mice were sensitized with DNFB. After 5 days, ears were challenged with DNFB and ear swelling was measured 24 hours later. *P<0.0005 versus Pos Co. Neg Co, negative control; Pos Co, positive control. (**b**) Lymph node cells and splenocytes were obtained from NP-injected mice (**a**, #3) and transferred intravenously (i.v.; 5×10^7 per mouse) into naive mice (Transfer) 24 hours before sensitization with DNFB or the irrelevant hapten oxazolone (Oxa). After 5 days, ears were challenged with the respective hapten and ear swelling was measured after 24 hours. *P<0.05 versus Pos Co; NS, not significant.

AhR, we utilized the AhR agonist 4-n-nonylphenol (NP; Bonefeld-Jørgensen *et al.*, 2007). The i.p.-injected NP induced the expression of the AhR-controlled enzyme CYP1A1 in lymph nodes as demonstrated by quantitative real-time PCR (data not shown), indicating that the dose injected activates the AhR. Mice were injected i.p. with NP on 4 days before sensitization. As observed for UVR, CHS was significantly suppressed by NP (Figure 3a; #3).

To analyze whether NP induces antigen-specific Tregs, lymph node cells and splenocytes obtained from DNFB-sensitized and NP-treated mice were injected into naive animals. After 24 hours, one group of recipients was sensitized with DNFB, and the other group with the unrelated hapten oxazolone (Oxa). Recipients sensitized with DNFB (Figure 3b; #3) were significantly suppressed in their ear swelling response, whereas recipients sensitized with Oxa revealed a pronounced CHS reaction (Figure 3b; #6). This indicates that NP induces Tregs that act in an antigen-specific manner.

NP-induced Tregs belong to the CD4$^+$CD25$^+$ subtype and express Foxp3

To analyze whether NP-induced Tregs belong to the CD4$^+$CD25$^+$ subset, mice were injected i.p. with NP before sensitization. After 5 days, lymph node cells and splenocytes were obtained from the NP-treated mice and separated into CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells. Both fractions were injected intravenously into naive recipients 24 hours before sensitization with DNFB. Mice injected with CD4$^+$CD25$^-$ cells (Figure 4a; #3) reacted with a pronounced ear swelling response similar to the positive control (Figure 4a; #1). CHS was significantly reduced in recipients receiving CD4$^+$CD25$^+$ cells (Figure 4a; #4), indicating that NP induces CD4$^+$CD25$^+$ Tregs.

Foxp3 is a key transcription factor in the development of natural CD4$^+$CD25$^+$ Tregs (Fontenot *et al.*, 2003; Hori *et al.*, 2003) and expressed by UVR-Tregs (Schwarz *et al.*, 2011). To clarify whether the same applies for NP-induced Tregs, we utilized DEREG (*DE*pletion of *REG*ulatory T cells) mice that express a diphtheria toxin (DT) receptor–enhanced green fluorescent protein under control of the *foxp3* gene locus (Lahl *et al.*, 2007). Thus, injection of DT results in the selective depletion of Foxp3-positive cells. DEREG mice were injected i.p. with NP on 4 days. After 24 hours, the last treatment animals were DNFB sensitized. After 48 hours, DT was injected for 3 days. At 5 days after sensitization, lymph node

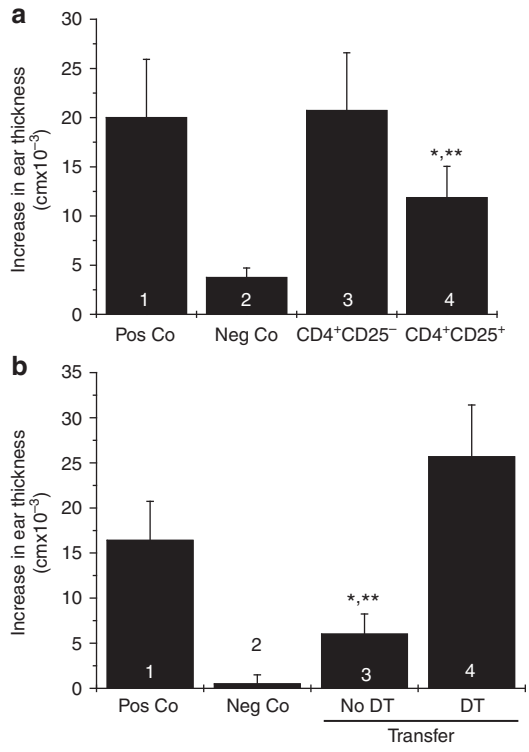

Figure 4. The 4-n-nonylphenol (NP) induces CD4$^+$CD25$^+$ regulatory T cells (Tregs) expressing forkhead box P3 (Foxp3). (**a**) Mice were injected intraperitoneally (i.p.) with NP for 4 days and sensitized with DNFB. After 5 days, lymph node cells and splenocytes were obtained and separated into CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells. CD4$^+$CD25$^-$ (#3) and CD4$^+$CD25$^+$ (#4) were injected intravenously (i.v.; 5×10^5 per mouse) into naive mice 24 hours before sensitization with DNFB. Ears were challenged and swelling measured. *$P<0.05$ versus Pos Co; **$P<0.005$ versus CD4$^+$CD25$^-$. Neg Co, negative control; Pos Co, positive control. (**b**) DEREG (*DE*pletion of *REG*ulatory T cells) mice were injected intraperitoneally (i.p.) with NP for 4 days. After 24 hours, mice were sensitized with DNFB. After 48 hours, one group (#4) received 1 μg diphtheria toxin (DT) on 3 days. At 5 days after sensitization, splenocytes and lymph node cells were obtained and injected i.v. into naive wild-type (WT) animals. Recipients were sensitized against DNFB 24 hours after injection, and were challenged 5 days later. *$P<0.0005$ versus Pos Co; **$P<0.000005$ versus DT.

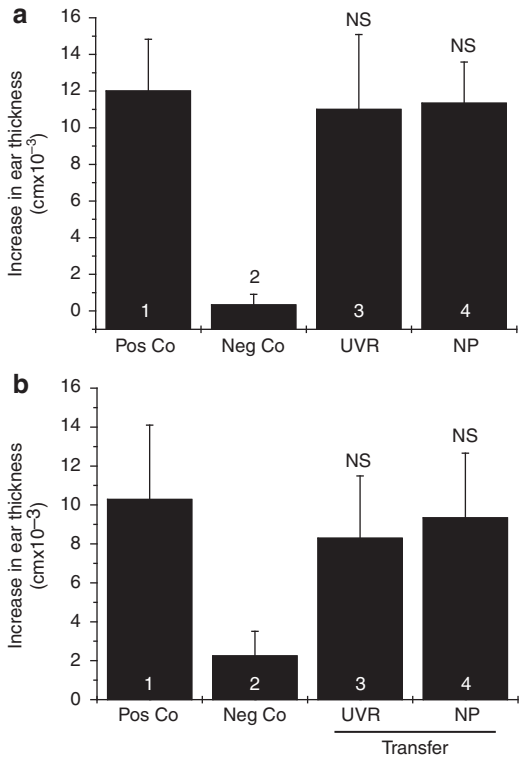

Figure 5. UVR and 4-n-nonylphenol (NP) do not induce immunosuppression in aryl hydrocarbon receptor knockout (AhR-KO) mice. (**a**) AhR-KO mice were UV irradiated (#3) or injected intraperitoneally (i.p.) with NP (#4) for 4 days. After 24 hours, mice were sensitized with DNFB. After 5 days, ears were challenged with DNFB and ear swelling was measured 24 hours later. Neg Co, negative control; Pos Co, positive control. (**b**) Lymph node cells and splenocytes were obtained from the UV-irradiated or NP-treated groups (**a**, #3 and #4) and injected intravenously (i.v.; 5×10^7 per mouse) into naive C57BL/6 mice (Transfer) 24 hours before sensitization with DNFB. After 5 days, ears were challenged with DNFB and ear swelling was measured after further 24 hours. NS, not significant.

cells and splenocytes were obtained and injected into naive wild-type mice. After 24 hours, the recipients were sensitized against DNFB and ear challenge was carried out 5 days thereafter. Cells obtained from DEREG mice treated with NP suppressed CHS in the recipients (Figure 4b; #3). In contrast, sensitization was not reduced upon transfer of cells obtained from DEREG mice treated with NP and DT (Figure 4b; #4), indicating that NP-induced Tregs express Foxp3.

AhR-KO mice are resistant to NP- and UVR-induced immunosuppression

To exclude that NP inhibits sensitization and induces Tregs through other mechanisms not involving the AhR, AhR-KO mice were injected with NP on 4 days before sensitization. After 5 days, ear challenge was carried out. NP significantly suppressed sensitization in wild-type but not in AhR-KO mice (Figure 5a; #4). Similarly, transfer of lymph node cells from AhR-KO mice into naive animals did not suppress sensitization of the recipients (Figure 5b; #4). This indicates that prevention of sensitization and induction of Tregs by NP involves the AhR.

We utilized the AhR-KO mice to further confirm the involvement of the AhR in UVR-induced immunosuppression. DNFB was painted on UVR-exposed skin of AhR-KO mice. In contrast to wild-type mice, AhR-KO mice turned out to be fully sensitized, despite the application of the hapten onto UVR-irradiated skin (Figure 5a; #3). Finally, splenocytes and lymph nodes cells were obtained from the UVR-exposed AhR-KO mice and injected intravenously into naive mice. After sensitization, the recipients responded to the challenge with a CHS response comparable to positive controls (Figure 5b; #3), indicating that UVR-Tregs had not developed in AhR-KO mice.

Injection of NP-treated dendritic cells induces Tregs

As the AhR appears to be involved in UVR-induced immunosuppression, the question arises as to which cell type needs to be activated by UVR through the AhR to initiate

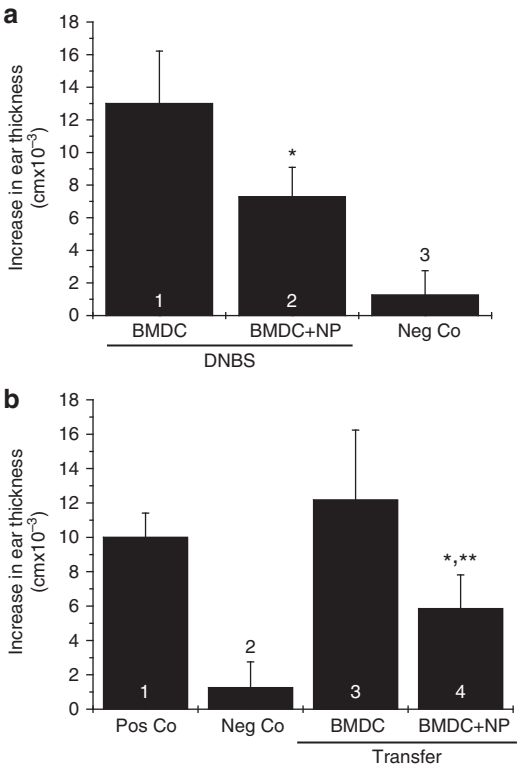

Figure 6. Injection of 4-n-nonylphenol (NP)-treated bone marrow–derived dendritic cells (BMDCs) induces regulatory T cells (Tregs). (**a**) BMDCs were incubated with (#2) or without NP (#1). After 8 days, mature BMDCs were harvested, pulsed with DNBS (1 mM for 30 minutes), washed, and injected subcutaneously (s.c.; 7×10^5) into naive mice. After 5 days, ear challenge against DNFB was carried out and ear swelling was measured. *$P<0.002$ versus BMDCs. Neg Co, negative control; Pos Co, positive control. (**b**) Lymph node cells and splenocytes were obtained from mice that had received untreated or NP-treated BMDCs (**a**, #1 and #2) and injected intravenously (i.v.; 5×10^7) into naive mice (Transfer, #3 and #4) 24 hours before sensitization. After 5 days, challenge was carried out and ear swelling was measured. *$P<0.002$ versus Pos Co; **$P<0.005$ versus BMDCs.

immunosuppression. As UVB does not penetrate beyond the upper dermis, most likely the candidates are located in the epidermis. As the AhR is ubiquitously expressed, exact identification will only be possible by transgenic mice in which the AhR is selectively knocked out in keratinocytes, Langerhans cells (LCs), or dermal dendritic cells. For UVR-induced immunosuppression, LCs appear to be required as depletion of LCs in Langerin DT knock-in mice was associated with a loss of UVR-induced immunosuppression (Schwarz *et al.*, 2010). Hence, we surmised that, rather than keratinocytes, dendritic cells are crucially involved in AhR-mediated immunosuppression. To further elucidate this phenomenon, we asked whether activation of the AhR in antigen-presenting cells induces immunosuppression. Thus, dendritic cells were obtained from the bone marrow (BMDCs), cultured in the absence or presence of NP, coupled with DNBS and injected subcutaneously into naive mice. Challenge with DNFB was carried out 5 days later. Mice injected with DNBS-coupled BMDCs responded with pronounced ear swelling (Figure 6a; #1). In contrast, upon injection of DNBS-coupled BMDCs, which were cultured with NP (#2), the recipients revealed a reduced ear swelling response. To analyze whether NP-treated BMDCs induce Tregs, lymph node cells and splenocytes obtained from donors (Figure 6a; #1 and #2) were injected into naive animals (Figure 6, Transfer) that were sensitized 24 hours later. Recipients of cells obtained from mice injected with NP-treated BMDCs were significantly suppressed in their ear swelling response (Figure 6b; #4), whereas recipients of cells from donors injected with the untreated BMDCs revealed a pronounced CHS reaction (Figure 6b; #3). These results imply that dendritic cells are essentially involved in AhR-mediated immunosuppression.

DISCUSSION

The major conclusions of this paper are 2-fold: first, the AhR is involved in UVR-induced immunosuppression; second, activation of the AhR induces similar immunosuppressive effects as observed with UVR.

The molecular mechanisms involved in UVR-induced immunosuppression appear to be complex. For a long time, it was assumed that UVR-induced DNA damage is the major, even the only molecular, event involved. This was based on the findings that reduction of DNA damage was associated with a mitigation of UVR-induced immunosuppression (Applegate *et al.*, 1989; Kripke *et al.*, 1992). This was confirmed by studies demonstrating that injection of mediators, which induce DNA repair such as IL-12, IL-18, IL-23 (Schwarz and Schwarz, 2009), and vitamin D (Halliday, 2010), reduced UVR-induced immunosuppression. As mostly reduction but not total prevention was observed, it was assumed that additional signaling pathways may be involved.

Cis–urocanic acid has been recognized rather early as an essential component involved in UVR-induced immunosuppression, as removal of *cis*–urocanic acid by tape stripping reduced immunosuppression (De Fabo and Noonan, 1983). Similar findings were obtained by the injection of anti-*cis*–urocanic acid antibodies (Beissert *et al.*, 2001).

On the basis of our data, the AhR can be added to the list of molecular targets that the UVR utilizes for exerting immunosuppression. This is indicated by the findings that AhR antagonists reduce UVR-induced immunosuppression and that AhR-KO mice are reduced in their immunosuppressive response to UVR. Initially, we used the AhR antagonist resveratrol that mitigated UVR-induced suppression. As resveratrol exerts additional activities including antioxidative effects (Afaq and Katiyar, 2011), we utilized the more specific AhR antagonist MNF (Lu *et al.*, 1995; Davis *et al.*, 2003). Prevention of UVR-induced immunosuppression by MNF and the fact that AhR-KO mice turned out to be resistant to UVR-induced immunosuppression indicates that the AhR is involved in the compromise of UVR to the immune system.

This is not surprising as it has been recognized that the function of the AhR goes beyond its detoxifying effects. Activation of the AhR induces transcription of various genes involved in the regulation of cell differentiation, proliferation, and activation (Schmidt and Bradfield, 1996). There is also evidence that activation of the AhR can modulate immune

responses (Kerkvliet, 2009). This was first indicated by the observation that benzo[a]pyrene induces hyporesponsiveness against DNFB (Ruby *et al.*, 1989). The AhR ligand TCDD was shown to induce Tregs (Funatake *et al.*, 2005; Marshall *et al.*, 2008; Quintana *et al.*, 2008). In turn, activation of the AhR by 6-formylindolo[3,2-b]carbazole inhibited development of Tregs and stimulated the differentiation of T helper type 17 cells (Quintana *et al.*, 2008). This indicated that AhR regulates both Tregs and T helper type 17 cell differentiation in a ligand-specific manner. The underlying mechanisms explaining these diverse effects still remain to be determined. The involvement of the AhR in T-cell development was further confirmed by the observation that activation of the AhR in the presence of transforming growth factor-β1 induced $Foxp3^+$ human Tregs (Gandhi *et al.*, 2010).

The AhR appears to influence maturation of LCs, as AhR-deficient LCs were smaller and less granular and did not upregulate costimulatory molecules (Jux *et al.*, 2009). Their phagocytic capacity was higher, another indicator of lower maturation. CHS to FITC was reduced in AhR-deficient mice. This is in contrast to our findings. Whether this is because of different types of haptens or concentrations applied remains to be determined. The impaired response in the presence of immature LCs appears to be surprising, as several studies have shown that LCs are not required for the induction of CHS (Romani *et al.*, 2010), but rather involved in downregulating immune responses including UVR-induced suppression of the induction of CHS (Schwarz *et al.*, 2010). In addition, immunosuppression does not appear to be the only UVR effect in which the AhR is involved, as UVR-mediated tanning was reduced in AhR-KO mice (Jux *et al.*, 2011).

As AhR is involved in UVR-induced immunosuppression, we studied whether similar immunosuppressive effects can be induced by AhR ligands. Because of its high toxicity, we avoided TCDD, which was shown to induce Tregs (Funatake *et al.*, 2005; Marshall *et al.*, 2008; Quintana *et al.*, 2008). NP is an alkylphenol interacting with the AhR (Krüger *et al.*, 2008). Its *in vivo* activity was proven by quantitative real-time PCR demonstrating the upregulation of CYP1A1 upon injection of NP. This was absent in AhR-KO mice (data not shown). Injection of NP inhibited the induction of CHS and induced Tregs. NP-induced Tregs act in an antigen-specific manner, as transfer of Tregs obtained from NP- and DNFB-treated donors inhibited the induction of CHS against DNFB but not against Oxa. Depletion studies revealed that NP-induced Tregs, such as UVR-Tregs, belong to the $CD4^+CD25^+$ subtype and express Foxp3 as demonstrated in DEREG mice. NP can interact with the estrogen receptor (Goksøyr, 2006; Krüger *et al.*, 2008). However, the effects of NP on CHS are not mediated through this pathway, as NP did not exert any immunosuppressive features in AhR-KO mice.

We surmised that activation of the AhR in dendritic cells is an essential event in AhR ligand-induced immunosuppression. This is based on the observation that injection of NP-treated and DNBS-coupled BMDCs rendered recipient mice unresponsive to DNFB. As LCs are crucially required for mediating UVR-induced local immunosuppression (Schwarz *et al.*, 2010), we speculate that cutaneous dendritic cells, most likely LCs, are those cells that, on activation of the AhR by UVR, mediate the inhibition of CHS and the induction of Tregs. However, we cannot definitely rule out that activation of the AhR in keratinocytes is involved as well. This issue could be ultimately clarified only by generating transgenic mice in which the AhR is selectively knocked out either in keratinocytes or dendritic cells.

In addition, we observed that Tregs require AhR to exert their suppressive features. The loss of suppressive activity caused by MNF was not due to the induction of cell death (data not shown), but associated with a downregulation of Foxp3. On the other hand, *in vitro* incubation of $CD4^+CD25^-$ T cells with NP did not shift them into a regulatory phenotype (data not shown). However, the limitation of this experiment is the fact that we may have not yet found the optimal conditions concerning concentrations or kinetics.

Taken together, our data suggest that AhR is involved in the induction of Tregs and in the maintenance of their suppressive activity. The first activity appears to be dependent on the activation of the AhR in dendritic cells, the latter in the Tregs themselves. By which ligands the latter effect is driven remains to be determined. Thus, our data identify the AhR as an additional molecular target involved in UVR-induced immunosuppression. Hence, the AhR appears as a potential target to induce antigen-specific immunosuppression. Activation of the AhR might represent an alternative to modulate the immune system in a similar manner like UVR, but without causing the adverse effects of UVR including DNA damage.

MATERIALS AND METHODS

Animals

Female, 7- to 8-week-old C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). AhR-KO mice (C57BL/6 were provided by J Krutmann (Schmidt *et al.*, 1996). DEREG mice were provided by T Sparwasser (Lahl *et al.*, 2007). To deplete Foxp3-positive cells, mice were injected i.p. with 1 μg DT (Merck, Darmstadt, Germany). Mice were bred in the central animal facilities of the University Clinics Schleswig-Holstein. Animal care was utilized by expert personnel under specific pathogen-free conditions, in compliance with relevant laws and institutional guidelines.

Reagents

As an AhR agonist, NP (Sigma-Aldrich, Taufkirchen, Germany) was used, and as an antagonist MNF (ICC Chemical Corporation, New York, NY) was used.

UVR-induced immunosuppression

Mice were irradiated with 1.5 $kJ\,m^{-2}$ UVR on their shaved backs for 4 consecutive days using fluorescent bulbs (TL12, Philips, Eindhoven, The Netherlands) that emit most of their energy within the UVB range. After 24 hours, the last exposure animals were sensitized by painting 50 μl of DNFB solution (0.5% in acetone/olive oil, 4:1) on the shaved back. After 5 days, ear challenge was carried out with 20 μl of 0.3% DNFB on the left ear and the ear swelling response was measured with a spring-loaded micrometer 24 hours later. For sensitization against Oxa, 100 μl of a 2% solution and for challenge 20 μl of a 1% solution were used. Positive control mice were sensitized and challenged, whereas negative control mice were only

challenged. The ear swelling response was determined as the difference in ear thickness of the hapten-treated ear compared with vehicle-treated ear and expressed in cm^{-3} (mean ± SD).

NP-induced immunosuppression

Mice were injected on 4 consecutive days with 200 µl 600 nM NP. After 24 hours, mice were sensitized, and 5 days later they were challenged.

Generation of Tregs

Mice were exposed to UVR or treated with NP. At 24 hours after the last treatment, animals were sensitized. After 5 days, lymph nodes and spleens were collected and single-cell suspensions were prepared. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated through magnet-activated cell sorter (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) using the $CD4^+CD25^+$ Regulatory T-cell Isolation Kit (Miltenyi Biotec). For adoptive transfer, lymph nodes cells and splenocytes obtained from donor animals (5×10^7) were injected intravenously into naive recipients. For adoptive transfer of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells, 5×10^5 cells were used. Recipients were sensitized 24 hours after injection and ear challenged 5 days later.

Generation of dendritic cells

BMDCs were generated by culture of bone marrow cells obtained from tibias (Schwarz and Schwarz, 2010). The expression of surface molecules characteristic for adult BMDC (major histocompatibility complex class II) was determined in representative samples by flow cytometry.

Statistical analysis

Statistical analysis was performed using Student's *t*-test. Differences between the means ± SD and the corresponding control value with $P<0.05$ were considered statistically significant. Unless otherwise stated, data show one representative of three independently conducted experiments with at least six mice per group.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We are grateful to Chris Opitz and Sarah Kersten for excellent technical assistance, and Arne Voss for help in preparing the graphs. This study was supported by grants from the German Research Foundation (SCHW625/4-2 and SCHW625/6-1).

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